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SOLUBLE SUBSTANCES AND ANTIGENS OF HUMAN EPIDERMIS (ANALYSES BY ELECTROPHORESIS AND IMMUNOELECTROPHORESIS)*

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Keratins are described as a class of fibrous proteins, sulfur cross-linked and insoluble. The study of solubilized wool fibers provides this understanding and other data related to their biochemical composition and properties (1). However, as a result of studies of the macromolecular structure of keratins of hair and of epidermis by electron microscopy (2, 3, 4) and by X-ray diffraction (1, 5), it has become apparent that keratin fiber evolves from several peptide components. Whereas some components appear to be synthesized by epidermal cells before reaching the keratogenous zone (6), others may form as the epidermal cell hardens (7, 8). It seems very likely that all prekeratinous components are present or anabolized by the epidermal cells in a soluble state and that their transformation into insoluble keratin is accompanied by the formation of soluble catabolites as by-products of the hardening process. In addition to the obvious importance of the soluble substances as metabolites in keratinization, recent reports indicate that they may also have a pathogenetic role of their own as auto-antigens (9, 10, 11).

The present work was undertaken to establish a baseline for further investigations of the nature and immunopathogenicity of solutes present in human epidermis and its horny derivatives. This report describes the preparation of extracts from normal epidermis and from its horny derivatives, the preparation of rabbit anti-epidermis serum and the results of electrophoretic and immunoelectrophoretic analyses—both followed by various methods of chemical characterization.

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MATERIALS AND METHODS

The following materials of epidermal origin were used in this study: solutes extracted from normal corporeal epidermis (referred to as cE, 6 different specimens), normal plantar epidermis (pE, 4 specimens), normal palmar epidermis (pE, 2 specimens), mucosal epidermis (mE, esophageal, 2 specimens and vaginal, 2 specimens) and epidermis of mature stillborn infants (6 specimens); solutes extracted from hyperkeratosis callus (HKK, 3 specimens) and from ichthyosis scales (ICHT, 3 specimens) were used to represent acellular horny products of plantar and corporeal epidermis respectively.

The normal corporeal, palmar and plantar epidermis was obtained at necropsies of patients who died of chronic heart disease (two), sequelae of liver cirrhosis (one), myocardial infarction (one) and accidental injuries (two). The skin surface was sponged with saline and wiped dry. A flat, square bottom of an enameled, metal tray filled with crushed dry ice-acetone mixture (−60° C) was pressed against the skin until the subcutaneous structures had frozen solid. Small areas of the frozen skin were warmed up (by pressing my palm against the frozen surface) long enough to thaw the epidermis, which was then scraped off with a sturdy spatula. The stillborn infants were preserved after necropsy frozen at −20° C, and the thawed epidermis was obtained by the same technic. Esophageal epidermis and vaginal epidermis were obtained from organs removed at necropsy or after hysterectomy and preserved at −20° C. Callus was obtained from 3 patients with congenital hyperkeratosis of palms and soles. Ichthyosis scales were collected over a period of several weeks from bed sheets of 3 patients with congenital ichthyosis. Two of these patients were siblings, 4 and 6 years of age. Individual specimens of the different types of epidermis were homogenized in a Virtis-45 homogenizer at −4° C for 30 min. at full speed in 0.2M sodium phosphate buffer pH 7.4. A ratio of 1 volume of tissue to 5 volumes of buffer was used. The homogenate was treated with ultrasound in a Raytheon sonic oscillator for 15 minutes. The sonicated homogenate was centrifuged twice at 40,000 g for 30 min. at 4° C. The floated lipids were removed. The sediment was subjected to a second extraction repeating the procedure just described. The supernatants obtained by the two extractions were pooled and dialyzed against continuously stirred distilled water for 18 hours at 4° C. A ratio of 1

volume of extract to 30 volumes of water was used for the dialysis. The dialyzed extracts were freeze-dried.

The callus shavings and the ichthyotic scales obtained from different patients were processed individually. They were first ground in a laboratory mill. The ground material was soaked for 24 hours in 0.2M sodium phosphate buffer pH 7.4 at 0° C, then homogenized (30 min., 0° C) and sonicated (15 min.). A ratio of 1 volume of tissue to 5 volumes of buffer was used for the extraction. The extraction procedure was repeated using sediment obtained after centrifugation (at 40,000 g/30 min./4° C). The supernatants of the two extractions were pooled, dialyzed against distilled water (1:30 v/v) and the non-dialyzable solutes freeze-dried.

The freeze-dried powders were stored at −20° C in airtight glass containers.

Extracts of human parenchymal organs were prepared from tissues obtained at necropsies; extract of white blood cells was prepared from buffy coats of heparinized blood of normal volunteers. The same technic as that used for extraction of epidermis was employed for extracting the solutes from these tissues.

Human lyophilized plasma, crystallized serum albumin, alpha globulins (Cohn's fraction IV), beta globulins (Cohn's fraction III), gamma globulin (Cohn's fraction II) and mercaptoalbumin were purchased from Mann Research Laboratories, Inc., N.Y.C. The mercaptoalbumin was prepared by crystallization of human serum albumin by mercuric chloride. The preparation reportedly contains besides serum albumin an albumin dimer, *i.e.*, two albumin molecules linked through one atom of mercury (12).

Normal human serum (referred to as NHS) was obtained by pooling fresh human sera of O, A, B and AB blood types. The pooled serum was dialyzed against 0.007M sodium phosphate buffer (pH 7.4) and freeze-dried.

Antisera against human epidermis were prepared by immunizing rabbits with pooled solutes extracted from corporeal, plantar, palmar and infant epidermis. Equal amounts of all extracts were pooled. Ten injections, each containing 25 mg of the pooled solutes dissolved in 3% sodium alginate adjuvant (13), the latter purchased from Colab, Inc., Chicago Heights, Ill., were given in weekly intervals subcutaneously to each rabbit. The immunization with epidermal solutes was followed by immunization with epidermal solids. The latter, packed by centrifugation from epidermal homogenates, were pooled, suspended in saline (in 1:10 v/v ratio) and rehomogenized (60 min., 0° C). Four injections, each containing 1 ml of the suspension and 1 ml of 6% sodium alginate were given in 10-day intervals subcutaneously to each rabbit. The animals were bled 4 to 8 weeks after the last injection. The individual antisera were assayed for precipitins against normal human plasma, against reconstituted NHS and against pooled epidermal solutes by double diffusion in agar. The antisera with the greatest variety of antibodies against epidermal solutes were pooled to obtain an optimally reactive anti-epidermis serum. The approximate equivalence-zone ratios of the pooled anti-epidermis serum to albumin and to γ globulin were established by test-tube precipitations (14). The antibodies against serum proteins, present in the anti-epidermis serum, were neutralized by the addition of appropriate amounts of the reconstituted NHS. The endpoint of absorption was controlled by double diffusion in agar against fresh human serum. The pooled anti-epidermis serum absorbed with serum proteins is referred to as *anti E serum.*

A portion of the anti E serum was heated for 60 min. at 56° C and absorbed with washed AB, Rhesus-positive erythrocytes.

The anti E serum absorbed with AB erythrocytes was used for assaying its immunological crossreactivity with solutes extracted from human parenchymal tissues.

Goat antisera against whole normal human serum (anti-HS serum) and monospecific antisera against individual human plasma proteins [goat anti-human-albumin, -α_2 macroglobulin, -γM globulin, -β_{1c}/β_{1a} globulin, -γA globulin, -γG globulin, -fibrinogen, -transferin, -orosomucoid, -β lipoprotein] were purchased from Hyland Laboratories, Los Angeles, Calif.

The extracts of epidermis and its horny derivatives were studied by horizontal starch gel electrophoresis (referred to as SGEP) (15), double immunodiffusion in agar (ID) (16, 17), immunoelectrophoresis (IEP) (18) and simple agar electrophoresis (AEP) (18).

For the SGEP, which employs a discontinuous buffer system, 10% and 15% (w/v) solutions of the freeze-dried extracts were used. The efficacy of the procedure is discussed in detail by Barret *et al.* (15). Some technical details and modifications used in this study are described below: Partially hydrolyzed potato starch gel (13 g/100 ml w/v) was prepared in pH 8 buffer made of 90 volumes of 0.016M Tris–0.033M citric acid and 10 volumes of 0.02M lithium hydroxide–0.076M boric acid. The starch suspension was agitated in a vacuum flask over open flame, until it became translucent gel. At its boiling point the gel was degassed under vacuum and poured into a preheated (80° C) Pyrex glass tray 25 x 14 x 0.25 cm. The glass tray was equipped with a false bottom cut from a flat acetate plastic sheet (0.0075 inch gauge) and made to adhere to the glass bottom by a thin layer of water-free glycerol. This plastic sheet bottom facilitated safe removal of the fragile starch gel from the tray and its transfer to the staining solution. After pouring, the gel was immediately covered with a preheated, oiled glass plate to insure uniform thickness of the starch sheet (2.4 mm). The gel was cooled at 4° C before inserting 20 x 2.3 mm filter paper strips soaked with the sample solution. During the electrophoresis the starch gel in the tray was covered with a ⅛ inch glass plate (23 x 14 cm)

glued to a plastic box filled with ice-salt mixture (−8° C). The cooling glass plate provided 1 cm of each end of the gel uncovered to receive flannelette wicks connecting it with the lithium-borate buffer in the electrode vessels (0.1M lithium hydroxide-0.38M boric acid, pH 8). The electrophoresis was carried out by maintaining a current of 42 milliamp. for 80–90 minutes, by continuously increasing the initial potential of 640 volts/25 cm of gel to 1120 volts. The horizontal LKB electrophoresis apparatus (3276 BN) without lid was used to support the starch tray and the LKB power supply (3371 C) to deliver the electric potential.

The undersurface of the starch gels was stained with 1% water-soluble nigrosin or with 3% amido black in methanol-glacial acetic acid-water mixture (50:10:40) for 30 to 60 minutes. The stained gels were left overnight in the first wash with the solvent, then washed by several changes of the solvent to produce an optimal contrast. Special staining procedures are described below.

A modified Ouchterlony technic was used for immunodiffusion in agar (ID): 2.5 mm thick, 0.7% agar in 0.05M veronal buffer of Michaelis type pH 8.6 containing 1:10,000 merthiolate was used. Five percent and 10% solutions (w/v) of the freeze-dried extracts were prepared in the same buffer. Two percent of normal human serum (17) was incorporated into the agar just before pouring it into Petri dishes at 55° C. The antiserum wells were refilled before the wells were completely empty until 0.5 ml of undiluted antisera were used up. The ID plates were kept at 37° C for 2 days, then washed in 3 changes of 1% NaCl solution (1:100 ratio of agar/saline) for 48 hours to remove the excesses of unprecipitated antisera and antigens. The saline wash was followed by washing in distilled water (1:100 ratio) for 2 hours to remove the NaCl from the agar. The lines of precipitates in the agar were first photographed; then the agar with the immunoprecipitate lines dried to a thin transparent film.

The immunoelectrophoresis (IEP) was carried out on the LKB electrophoresis apparatus. Five percent, 10% and 15% solutions of the freeze-dried extracts were made in 0.025M veronal buffer pH 8.6. The electrophoresis of 5 mcl samples was carried out under a potential of 8 volts/1 cm of agar for 50 minutes. After completion of the electrophoresis of the samples, the troughs were filled with 0.15 ml of antisera and incubated overnight at 37° C. A modification of the immunoelectrophoretic technic, namely the "short trough" technic (19) was employed to examine the antigenic relationships of some components present in complex electrophoretic patterns.

The unprecipitated antisera and antigens and the salts were removed from the agar by washing with 1% saline and with distilled water. The immunoprecipitate arcs were first photographed, then the agar with the immunoprecipitate arcs dried to a thin transparent film.

The preparation of solutions of freeze-dried extracts and the simple agar electrophoresis technique (AEP) was the same as that employed for IEP, except that instead of allowing formation of immunoprecipitate arcs by diffusion of antisera against the electrophoretically resolved components, the agar slides after electrophoresis were instantly immersed for 18 hours into a fixative of 2% glacial acetic acid or into 2% glacial acetic acid in 60% ethanol, followed by a 2-hour wash in distilled water. The agar layer containing the electrophoretically resolved and immobilized substances of the extracts was then dried to a thin transparent film.

The sample solutions of the freeze-dried extracts of epidermis and its horny derivatives, made in 5%–15% concentrations (w/v) in various buffers, were prepared fresh for each experiment. They were manually stirred at room temperature for 1 hour, then left overnight at 4° C, and just before use centrifuged at 10,000 g at −10° C for 5 to 45 minutes to free the samples of undissolved sediment and of lipoid substances.

The following special staining methods were applied to the starch gels as characterization reactions for the components of the extracts resolved by the SGEP: the method of Barrnett and Seligman (20) which employs 2,2′ dihydroxy 6,6′ dinaphthyl disulfide and tetrazotized diorthoanisidine (referred to as DDD reaction) and the method of Bennett (21, 22) which employs 1-(4 chloromercuryphenylazo) naphtol-2, *i.e.* Mercury Orange (referred to as RSR* reaction) were used for detection of protein-bound sulfhydryl, and the method of Barrnett and Seligman (23) utilizing 3,3′ dianisole bis 4,4′-3,5-diphenyl tetrazolium chloride, *i.e.* Blue Tetrazolium (referred to as ATZ† reaction) capable of reacting with sulfhydryl and disulfide groups of proteins but reportedly not of ultimate specificity (24, 25, 26).

For the DDD reaction the unfixed starch gel sheets were immersed after electrophoresis into solution containing 50 mg DDD per 100 ml of absolute ethanol and 0.1M veronal buffer pH 8.6 (1:1) and incubated at 50° C for up to 60 minutes. After the incubation the starch sheet, rinsed with water, was washed for 10 minutes in acidified water and in 65% and in 95% ethanol and in absolute ether, 3 minutes each wash. After a rinse with water the starch sheet was immersed into freshly prepared solution of 0.1% tetrazotized o-dianisidine in pH 7.4, 0.2M phosphate buffer or in 2M sodium acetate.

For the RSR reaction unfixed starch sheets were immersed after electrophoresis into a solution of 10^{-4} M Mercury Orange (M.W. 483.4) in dimethyl formamide (at 4° C) or in 80% ethanol (27) for 5 to 16 hours.

For the ATZ reaction unfixed or fixed (60% ethanol/15 min.) starch sheets were immersed in 0.1% Blue Tetrazolium salt in 0.1M, pH 12 glycine buffer (26) and incubated at 50° C for 30–120 minutes.

The following special staining methods were

* Red Sulfhydryl Reagent (26).
† Alkaline TetraZolium (26).

applied to characterize the epidermal substances precipitated in agar by anti E serum (by ID and by IEP), or immobilized in agar by a fixative (after AEP):

Aqueous solution of 1% amido black was used for general staining of proteins; saturated solution of Oil red O in 60% ethanol (28) was used for staining of lipids and of lipoproteins; staining by pyronine Y (G.T. GURR, London) was used for staining of polynucleotides; periodic acid-Schiff reaction (PAS), periodic acid-naphthol-p-phenylendiamine reaction (PA-Nadi) and periodic acid-tetrazotized o-dianisidine reaction (PAF*) were all utilized to demonstrate the presence of chemical groups capable of being oxidized by periodic acid. All of the above enumerated characterization reactions were executed as described in detail by Uriel (29) except for the reactions employing periodic acid; in the latter, the length of time allowed for oxidation of immunoprecipitates by periodic acid was shortened to 1–3 minutes.

The DDD, RSR and ATZ reactions were also used for staining of the immunoprecipitates and of components fixed in agar after AEP. The same technic as that used for the starch gel sheets was used for the dried agar films.

RESULTS AND COMMENTS

Starch Gel Electrophoresis (SGEP)

Fractions of human serum and whole serum were used in preliminary experiments to establish the regions of separation and subdivision of their components. By SGEP, performed under the conditions later employed for investigation of the various extracts of epidermis and its horny derivatives, albumin advanced in the starch gel 6.5–8.0 cm from the origin toward the anode, producing a single, solid, elipsoid blot; the same was true of the albumin component of the mercaptoalbumin. Alpha globulins advanced 5.5–6.5 cm toward the anode resolving as poorly staining, thin bands. The components of the beta globulins advanced no farther than 5.0 cm from the origin toward the anode. The mass of gamma globulin did not produce a defined band but advanced by electrophoresis toward the cathode producing a trail 2.5 cm long (Fig. 1). Accordingly, the pattern produced by SGEP of whole human serum was divided into 4 regions and the zones designated as follows: albumin zone (6.5–8.0 cm from the origin), A zone (5.5–6.5 cm from the origin) and B zone (0–5.0 cm). The cathodic trail of γ globulin, 2.5 cm long, along with region beyond it was termed zone C.†

One extract from hyperkeratosis callus termed HKK_1 was used as an arbitrary scale for SGEP patterns obtained with other extracts, because its numerous substances separated by the SGEP with remarkable clarity and constancy (Fig. 2, Fig. 3 and Fig. 4). Its main components were designated according to the zones into which they advanced and numbered from origin toward the anode as follows: 4 components within the B zone as B_1 through B_4, 2 components within the A zone as A_1 and A_2, 3 components within and one beyond the albumin zone as post-albumin (pA), intra-albumin (iA), albumin (Al) and pre-albumin; 4 components which advanced in the direction of the cathode and appeared as solid bands were designated as C_1 to C_4 and are referred to as post γ globulin components (Fig. 2).

The patterns (*i.e.*, the number of compo-

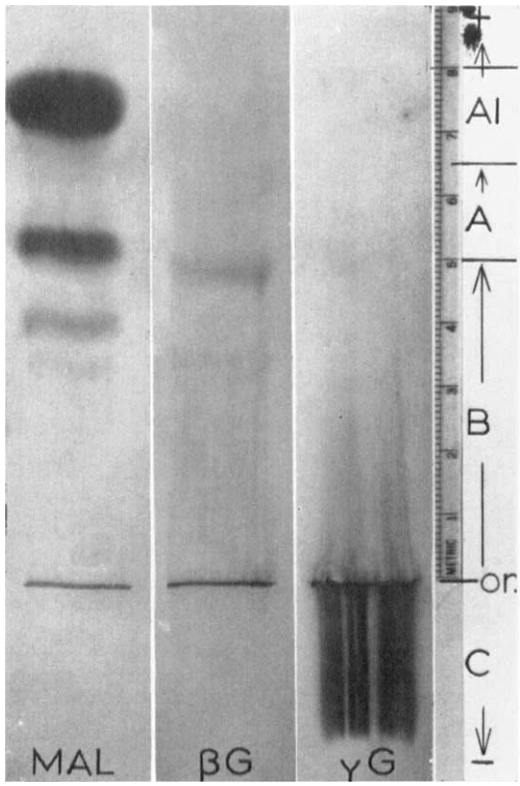

FIG. 1. Starch gel electrophoresis of 2% mercaptoalbumin (MAL), of 2% β globulins (βG) and of 4% γ globulin (γG). The regions to which the serum proteins advanced from the origin (or.) toward the anode were designated albumin zone (Al) (6.5–8.0 cm from the origin), A zone (5.5–6.5 cm from the origin) and B zone (0–5.0 cm); the region from the origin toward the cathode (0–2.5 cm and beyond) was termed zone C.

* Periodic acid-Formazan (29).

† Substances present in the extracts of epidermis or horny derivatives which separated within zone C as bands are referred to as post γ globulin substances or components.

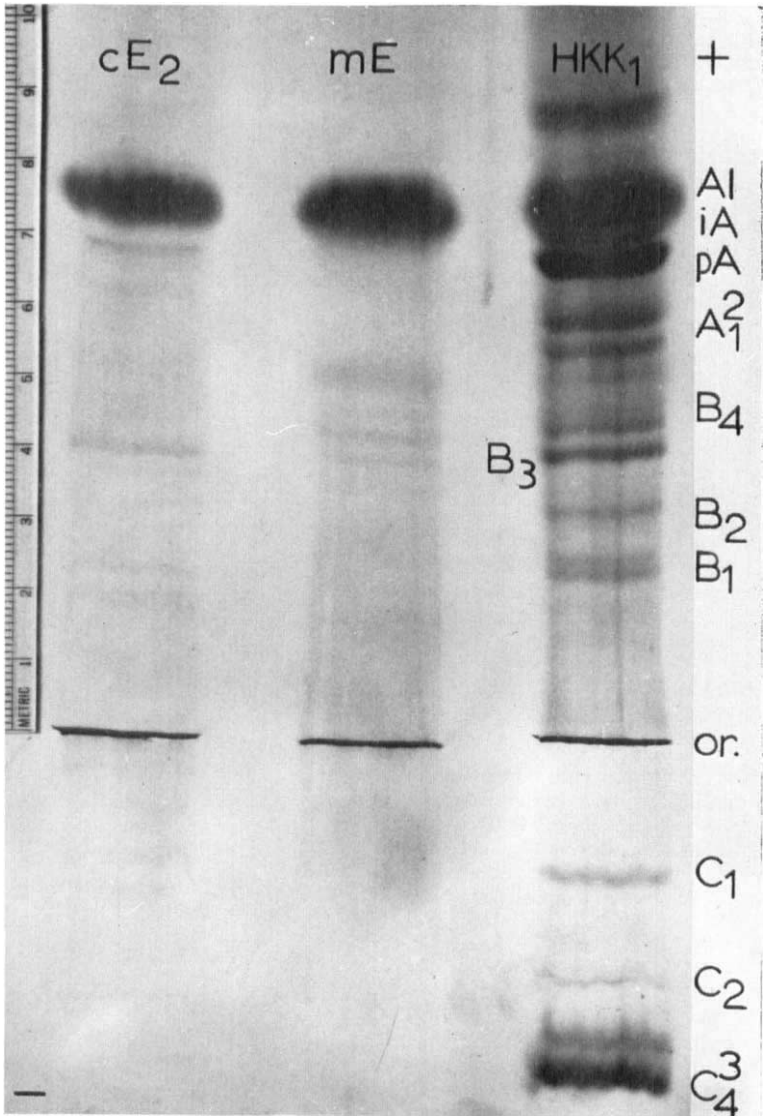

FIG. 2. Starch gel electrophoresis of solutes extracted from corporeal epidermis (cE_2, 10%), from mucosal epidermis (mE, 10%) and from hyperkeratosis callus (HKK_1, 15%). The HKK_1 extracts was selected to represent a relative scale for other extracts because its numerous substances separated with remarkable clarity and constancy. The resolved components were designated according to the zone into which they advanced and numbered as shown above: B_1 through B_4, A_1 and A_2, post albumin (pA), intra-albumin (iA), albumin (Al) and pre-albumin. Those components which advanced toward the cathode were designated C_1 through C_4 and are referred to as post γ globulin components.

nents and their relative positions) obtained by SGEP of different extracts of normal palmar and plantar epidermis (pE) closely resembled each other as well as the pattern of HKK_1. The substances of the albumin zone, of the A and of the C zones present in the pE extracts seemed to correspond with those present in HKK_1; however, the substances of the B zone appeared to differ: only 3 substances of the pE extracts advanced into the B zone and only 2 seemed to correlate with the B_1 and B_3 components of the HKK_1 (Fig. 3). It is worthy of note that the B_1 component of all HKK extracts resolved into 2 very closely spaced bands.

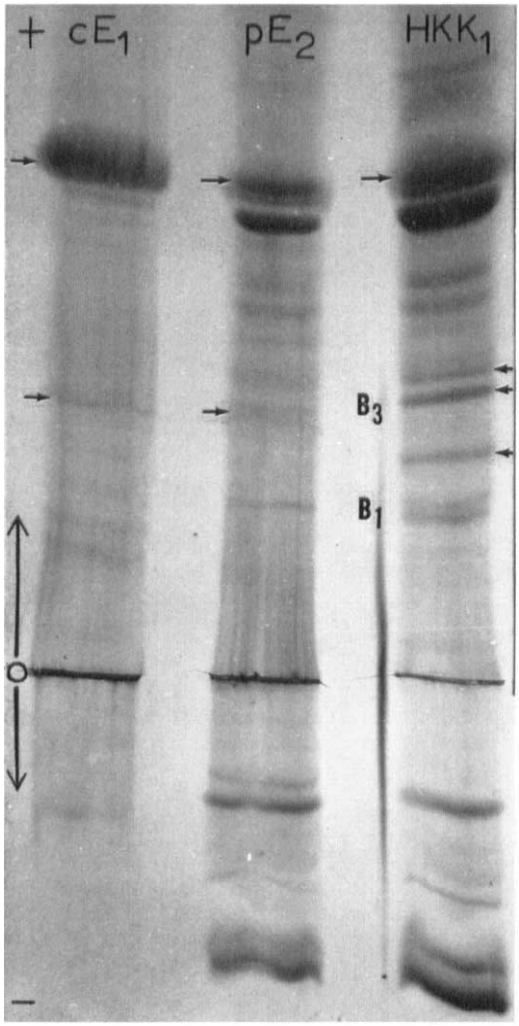

FIG. 3. Starch gel electrophoresis of solutes extracted from corporeal epidermis (cE_1, 10%), from plantar epidermis (pE_2, 10%) and from hyperkeratosis callus (HKK_1, 10%). The arrows show the components carrying sulfhydryl groups.

The correlated component of all pE patterns appeared single.

The patterns obtained by SGEP of corporeal epidermis (cE) were at variance with those of pE and of HKK_1 (Fig. 2 and Fig. 3). Most apparent was the relative dearth of substances trailing albumin (iA, pA) and of the post γ globulin components. The number and the mobilities of the components resolved within the B zone varied even among the individual cE patterns. However, one substance appeared in all of the different cE extracts. The electrophoretic mobility of this component corresponded to that of a B zone component of all pE extracts, and to that of the B_3 component of the HKK_1 (Fig. 3). In some cE and pE extracts this component resolved into 2 very closely spaced or intertwined lines (Fig. 3).

The patterns obtained by SGEP of mucosal epidermis (mE) lacked the albumin-trailing components (iA, pA). Only a trace of a post globulin band appeared after prolonged staining. Two components resolved within the B zone and appeared to have the mobility of the B_3 and B_4 components of the HKK_1 (Fig. 2).

The above comparisons of the SGEP patterns relate the most frequent features of the individual patterns. Fig. 2 and Fig. 3 portray

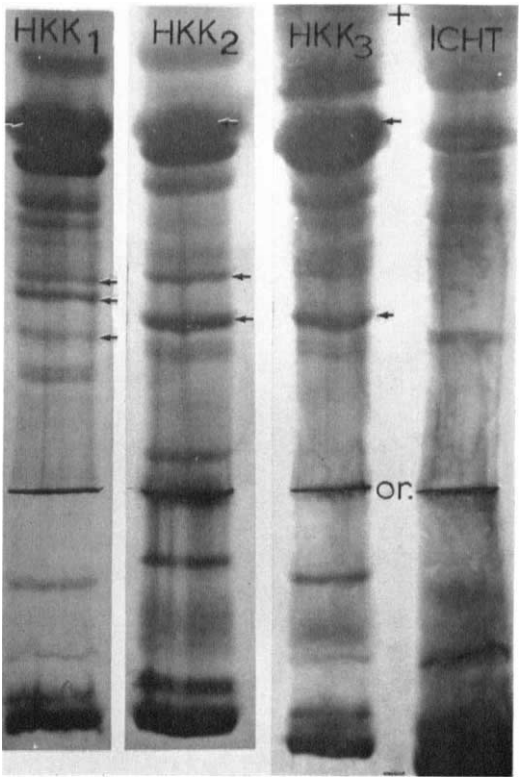

FIG. 4. Starch gel electrophoresis of solutes extracted from hyperkeratosis callus obtained from 3 patients (HKK_1, HKK_2 and HKK_3, all 15%) and of solutes extracted from ichthyosis scales (ICHT, 15%). The arrows point to components carrying sulfhydryl groups. Only one, the most anodically advanced component resolving in the albumin zone of the HKK patterns was endowed with sulfhydryl groups. No reactive sulfhydryl groups were detected in ICHT extracts.

these average features. However, some divergencies from the average were observed in the cE patterns. Description of these is beyond the scope of this report. The most marked divergence was encountered among the SGEP patterns of the callus extracts obtained from 3 patients. The most striking variations occurred among the substances resolved in the B zone, whereas those advancing into the albumin, A and C zones were comparable (Fig. 4). A typical pattern obtained by SGEP for extracts of ichthyosis scales (ICHT) is shown in Fig. 4. In contrast to the HKK patterns, the ICHT patterns were characterized by the relative scarceness of substances advancing into the albumin zone and the relative excess of substances advancing against the cathode. One component only resolved within the B zone and appeared to relate to the B_1 components of the HKK patterns.

In an attempt to identify the components among those resolved by the SGEP, the DDD, the RSR and the ATZ staining reactions for protein-bound sulfhydryl groups were employed. Positive results were obtained with all: the substance of the B_3 component of the HKK_1 gave a strongly positive DDD reaction. The components termed as B_2 and B_4 stained faint to moderate. (Only 2 components of the HKK_2 and 1 component of the HKK_3 gave a positive DDD reaction, see Fig. 4.) One component of the B zone of 3 out of 6 extracts of cE and of 3 out of 5 extracts of pE gave faint to moderate DDD reactions. This reacting component corresponded to the B_3 component of the HKK_1 (Fig. 3). No reaction was obtained with mE or with ICHT extracts. The albumin component (Al) of all extracts except those of ICHT gave a moderate DDD reaction, whereas the intra-albumin (iA) and post-albumin (pA) components of all HKK, pE and cE extracts did not react. Pre-treatment of the starch gels with 0.1M N-ethyl maleimide or with 0.1M iodoacetate prevented a subsequent positive DDD reaction, presumably due to an irreversible block of the sulfhydryl groups (26).

Only those components which stained intensely by the DDD method have stained distinctly by the RSR method. Others blended into the color of the starch gel. Distinct RSR reactions were obtained only with the extracts of HKK and with 2 out of 3 extracts of pE.

The ATZ reagent stained intensely all of the B zone components—as did the DDD reaction; albumin gave a strongly positive reaction, the intra- and post-albumin components did not react at all. In the HKK_1 pattern the closely spaced substances of the B zone diffused into each other before staining was accomplished, preventing identification of the unstained components; however, meaningful information was obtained by staining of the SGEP patterns of the cE and pE extracts: in all of the latter extracts only one component of the B zone—that which correlated with the B_3 component of HKK_1—reduced the ATZ reagent and stained. Two of the post γ globulin components of all extracts reacted with ATZ, though the stain was faint and diffused rapidly.

Fresh human serum (8% protein), 2% solution of Cohn's serum fractions II, III and IV and 1% albumin resolved in starch gel were subjected to the DDD, RSR and ATZ reactions; except for albumin none of the serum components or fractions stained.

In preliminary experiments, the rabbit anti E serum, absorbed with AB-Rh-positive erythrocytes after its antibodies against serum proteins were neutralized, was tested for immunological cross-reactivity with solutes present in extracts of visceral organs. One percent to 15% solutions (w/v) of the latter extracts were used for immunoelectrophoresis (IEP) and for immunodiffusion (ID). The most pronounced cross-reactions occurred with the extracts of striated muscle, white blood cells and spleen. Lesser cross-reactions occurred with extracts of lung and intestinal smooth muscle, minimal with extracts of liver and kidney, none with extracts of collagenous tendons. The types of the cross-reactions produced by the first 4 extracts, enumerated above, are shown in Fig. 5.

The cross-reactions were further examined by ID using 3 types of antisera: the anti E serum, the anti E serum absorbed with erythrocytes and anti E serum absorbed with individual organ extracts. The results made it apparent that the cross-reactions of the various substances in the organ extracts are due to their partial antigenic relationship to antigenic determinants carried by at least 3 substances present in the extracts of epidermis. The IEP and ID patterns obtained with anti E serum and with anti E serum absorbed with erythrocytes were the same; absorption of the anti E sera with any one organ extract did not affect its reactions with the extracts of epidermis, but it did not neutralize its cross-reactivity with all other organ extracts. Because of these observations it was elected to use the anti E serum for the immunochemical studies without neutralization of its cross-reactivities, rather than to risk introduction of unrelated, bio-

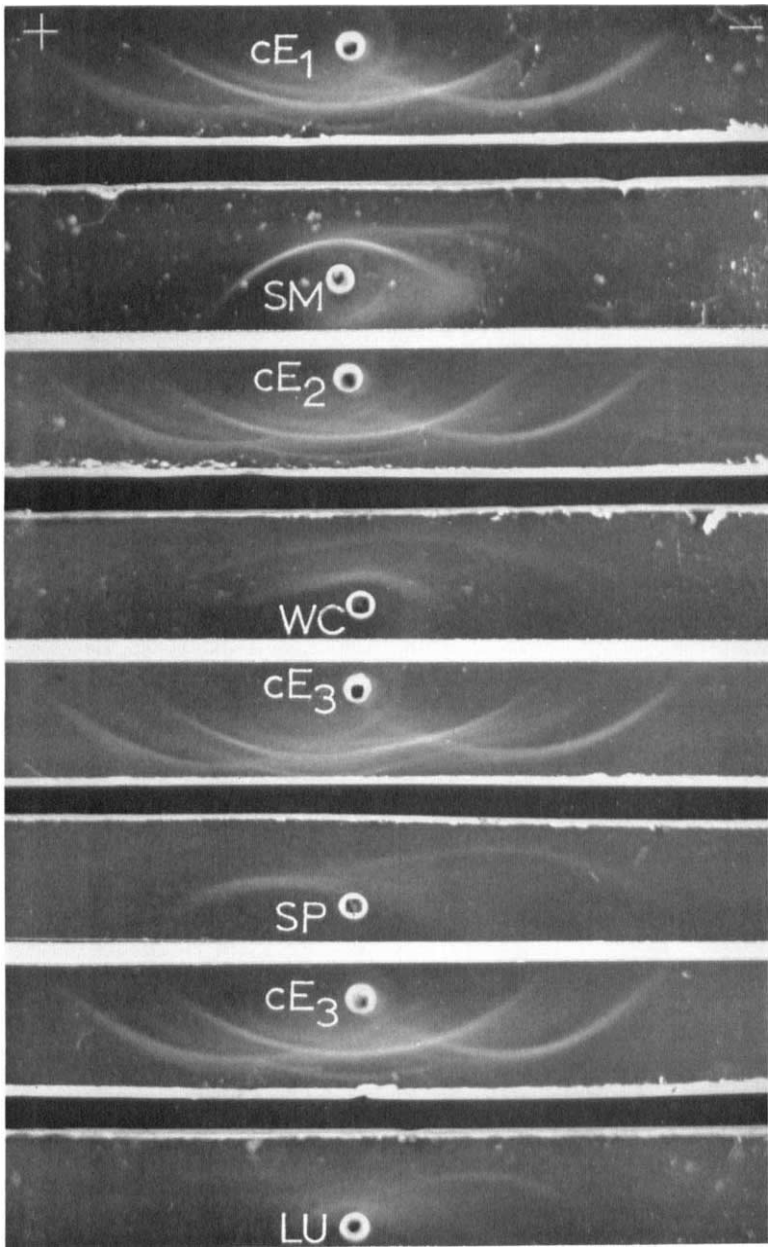

FIG. 5. Immunoelectrophoretic patterns obtained with solutes extracted from 3 different specimens of corporeal epidermis (cE_1, cE_2 and cE_3, all 10%) and solutes extracted from striated muscle (SM), from circulating white blood cells (WC), from spleen (SP) and from lung (LU). The precipitin arcs of all patterns were developed with anti-epidermis serum absorbed with serum proteins and with AB, Rh-positive erythrocytes.

chemically active substances of the organ extracts into the antiserum.

The biochemical significance of the cross-reactions of the anti E serum with substances present in the extracts of visceral tissues was not fathomed.

Immunodiffusion (ID)

Most of the individual extracts of the different types of epidermis (*i.e.*, cE, pE and mE) and of the 2 types of its horny derivatives (*i.e.*,

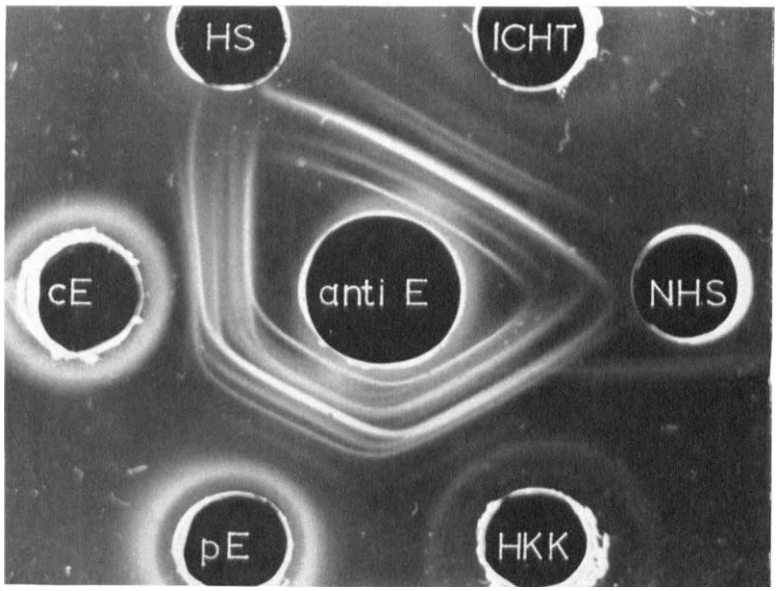

FIG. 6. Immunoprecipitates developed in agar by double diffusion of solutes extracted from corporeal epidermis (cE, 10%), from plantar epidermis (pE, 10%), from hyperkeratosis callus (HKK, 10%) and from ichthyosis scales (ICHT, 10%) against anti-E(pidermis) serum (anti-E). Reconstituted normal human serum (NHS, 5%) and undiluted fresh human serum (HS) were used as controls.

HKK and ICHT) were reacted against the anti E serum. Numerous precipitin lines developed against all extracts. The number of lines, the reactions of identity, the patterns of splitting and the density of precipitation produced by some components were fairly characteristic for each type of epidermis and the horny derivatives as well (Fig. 6).

Experiments were set up to test whether or not all of the antigenic substances are present in all of the types of extracts. The results showed that all of the antigenic components of pE, although in greatly differing amounts, are represented in cE, in HKK and in ICHT. The extracts of mE were the only exception; two extracts of the latter, one from vaginal, the other from esophageal epidermis, lacked one component present in all other extracts.

Immunoelectrophoresis (IEP)

The individual extracts of cE and pE were examined by IEP using anti-HS serum, monospecific antisera against individual plasma proteins and anti E serum. The presence of plasma proteins in the different cE and pE extracts varied quantitatively and qualitatively. Albumin, transferin, γ globulin (γG) and traces of β globulins were present in all cE and pE extracts, but up to 7 different plasma proteins were detected in 2 of the 6 different extracts of cE (Fig. 7).

Immunoprecipitate arcs, developed by interaction of the anti E serum with the antigenic substances of epidermal origin—immunologically unrelated to human plasma proteins, produced patterns characteristic for each type of extract. To facilitate the description of the patterns, the positions of the immunoprecipitate arcs produced by interaction of the epidermal antigens and anti E serum were related to the positions of the immunoprecipitate arcs produced by interaction of the serum proteins present in the epidermis and the anti HS serum. The former arcs were then termed according to the region they would occupy within the latter pattern (illustrated in Fig. 7).

The cE was characterized by a pattern consisting of 4 distinct arcs: one in the post-albumin region, 2 parallel arcs in the trans β region and one in the post γ globulin region. A third precipitin arc developed in the β_2 region only with some cE extracts (pattern A in Fig. 8). The latter β_2 region arc was prominent in the pE patterns and together with several post

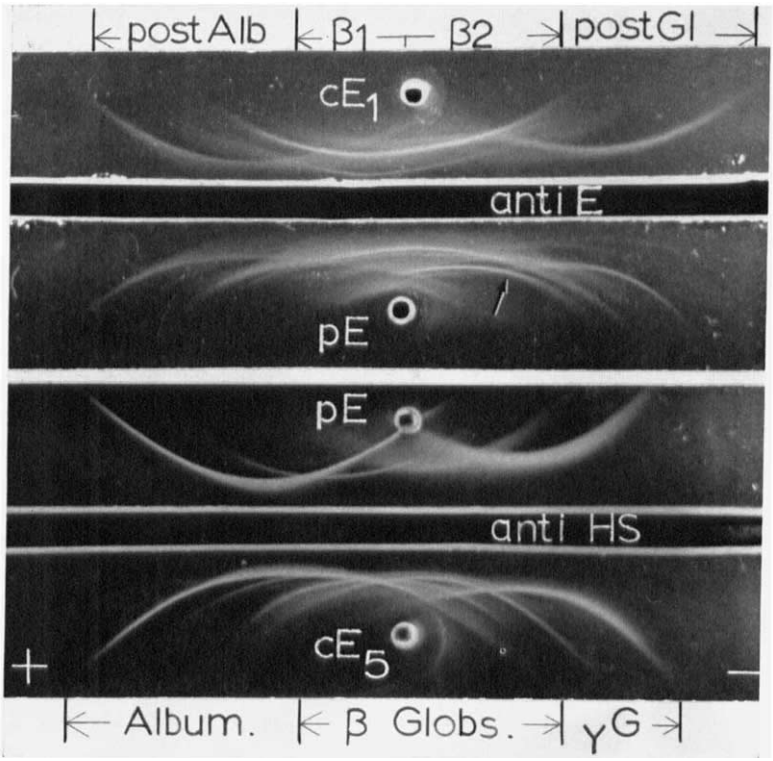

FIG. 7. Immunoelectrophoretic patterns obtained with solutes extracted from corporeal epidermis (cE_1, 10% and cE_5, 15%) and from plantar epidermis (pE, 10%). The upper patterns were developed with anti-E(pidermis) serum (anti-E) whereas the lower patterns were developed with anti Human-Serum serum (anti-HS). The terms used for designation of the immunoprecipitate arcs produced by the unknown epidermal antigens were termed according to the position they would occupy among the immunoprecipitate arcs produced by the serum proteins present in the epidermis. The former immunoprecipitate arcs were designated as follows: post-albumin (region) arc, trans β region arc(s), post γ globulin (region) arc(s) and β_2 region arc(s), the latter shown by an arrow.

γ globulin region arcs distinguished the patterns of palmar and plantar epidermis from those of corporeal epidermis (compare patterns B and A in Fig. 8).

Common antigenic determinants of the substances present in cE and pE and those present in HKK, ICHT and mE were demonstrated by the ID studies described above. These observations were extended by immunoelectrophoretic analysis: a characteristic pattern for each type of extract developed with the anti-E serum is shown in Fig. 8.

The HKK patterns were characterized by development of 2 to 4 parallel trans β region arcs and by 2 closely spaced arcs in the β_2 region (pattern C, Fig. 8). The immunologic identity of the latter arcs with the β_2 region arc of the pE patterns was established by IEP using the "short trough" technic. All other β region arcs of the HKK, pE and cE shared common antigenic determinants.

The ICHT patterns having 2 trans β region arcs resembled the patterns of cE (pattern D, Fig. 8). However, the arc near the antigen well was found immunologically related to the β_2 region arcs of the pE and of HKK.

The mE extracts produced patterns similar to cE, but characteristically lacked the arc of the post-albumin region—present in all other extracts. Traces of the post γ globulin region arc developed in the extracts of vaginal epidermis only (pattern E, Fig. 8).

The immunological identity of the substances responsible for the post-albumin region arcs of the different extracts was verified by the "short trough" IEP; the antigenic relationship

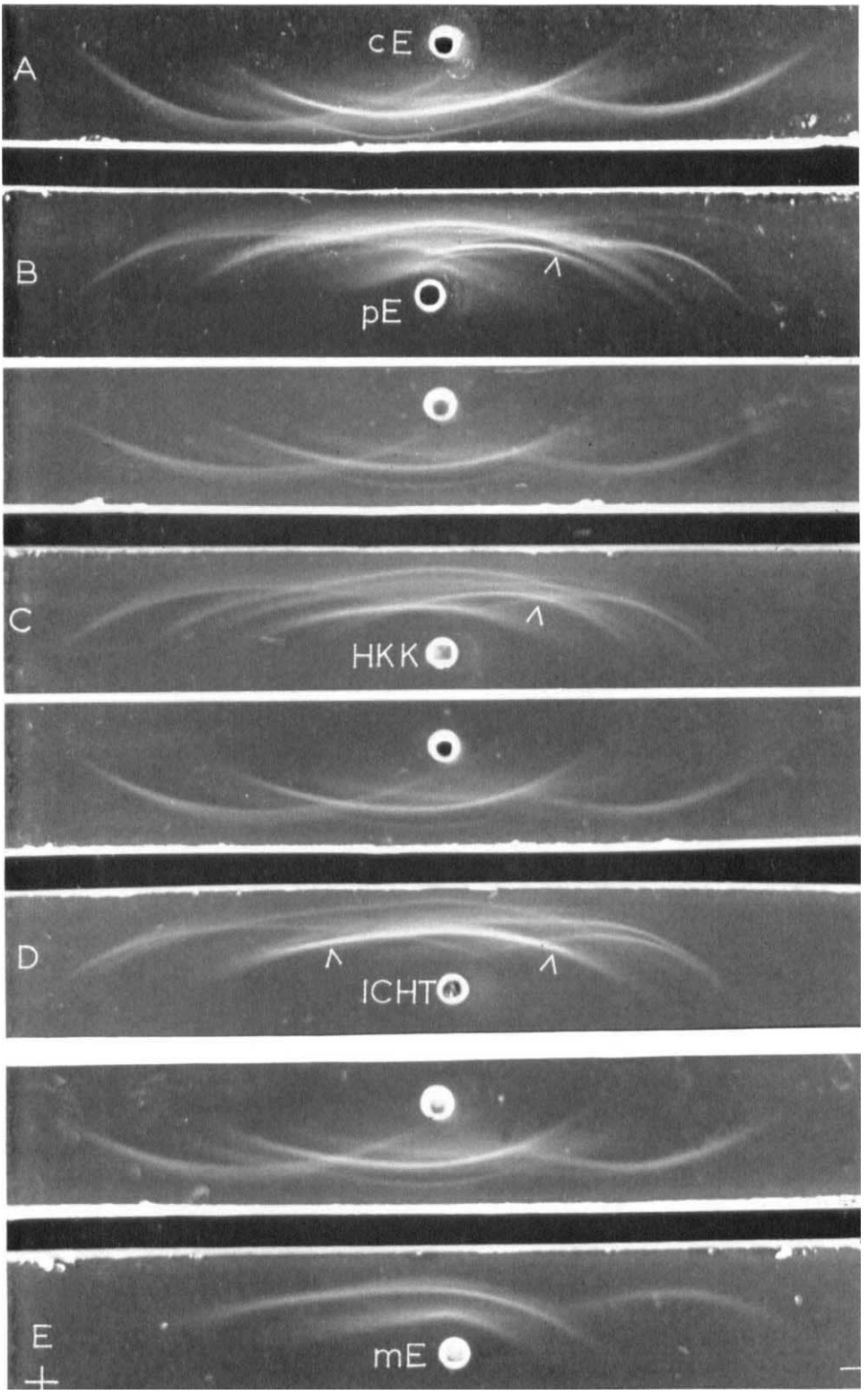

FIG. 8. Immunoelectrophoretic patterns obtained with solutes extracted from corporeal epidermis (cE, 10%), from plantar epidermis (pE, 10%), from hyperkeratosis callus (HKK, 10%), from ichthyosis scales (ICHT, 10%) and from mucosal epidermis (mE, 15%). The precipitin arcs were developed with anti-E(pidermis) serum. Arrows point to an immunoprecipitate present in extracts of pE, HKK and ICHT termed β_2 region arc. The latter precipitates gave positive reactions for carbonyl groups liberated by oxidation with periodic acid.

of the post γ globulin components was confirmed by the same technic.

Chemical characterization procedures were used to investigate the nature of the immunoprecipitates obtained by IEP and ID. The reactions of the immunoprecipitates with the various reagents were controlled by simultaneous processing of agar films obtained by simple AEP of the extracts.

The DDD and the RSR reagents failed to stain the immunoprecipitates although positive and specific staining reactions were obtained with the extracts of cE, pE and HKK resolved in agar by simple AEP. These contradicting results indicate that even though the components with sulfhydryl groups were present in the agar after electrophoresis, they could not be demonstrated after the reaction with the antiserum. The ATZ reagent did stain the immunoprecipitates but only after a prolonged time was allowed for the reaction. The intensity of the staining of the individual precipitin arcs varied markedly, but was not related to the amounts of precipitation. The trans β region arcs of the cE, pE and HKK extracts stained with maximal intensity. The same results were obtained by staining of precipitin lines produced by ID. The most plausible explanation of the lack of sulfhydryl reaction of the immunoprecipitates with the DDD and the RSR reagents is that the sulfhydryl groups were blocked by the antibody, being part of the site of the antigen-antibody union. It would appear that only the prolonged incubation at pH 12 with ATZ accomplished sufficient separation of this union to permit a reaction of the sulfhydryl or disulfide bonds with the stain.

Three reactions (PAS, PA-Nadi and PAF) which employ oxidation by periodic acid followed by stains capable of detecting liberated carbonyl groups were applied to the immunoprecipitates. All of the β region arcs gave positive reactions of varying intensities, especially in the PAS reaction. However, only the β_2 region arcs of the pE, HKK and ICHT patterns remained positive with all 3 staining procedures after the length of time allowed for oxidation by periodic acid was reduced.

Characterization reactions employed to demonstrate the presence of lipids, lipoproteins and polynucleotides failed to demonstrate such substances among the immunoprecipitins.

DISCUSSION

Two electrophoresis technics, that is, immunoelectrophoresis (IEP) and starch gel electrophoresis (SGEP), both capable of a high degree of resolution and separation of peptides, were employed for comparative study of soluble substances extracted from human corporeal, palmar, plantar and mucosal epidermis and of substances extracted from acellular callus and from scales of ichthyosis patients. Histochemical methods of staining were used for characterization of the substances resolved by the two technics. The substances present in these extracts resolved into well-defined, narrow bands by electrophoresis in starch gel or as immunoprecipitate arcs by immunoelectrophoresis. The positions of these bands and arcs were compared to positions of bands and arcs produced by known serum proteins, and the terms used for description of the electrophoretic mobilities of the latter were adapted to describe those of epidermal origin.

By means of IEP, at least 5 substances carrying different antigenic determinants were demonstrated in extracts of normal corporeal, palmar and plantar epidermis, as well as in extracts of acellular callus and scales. The antigenic determinants of these substances were different from those of normal plasma proteins. The patterns of the immunoprecipitate arcs obtained with individual extracts of any one type of epidermis (*i.e.*, cE, pE, mE) or its horny derivatives (*i.e.*, HKK, ICHT) were nearly identical, making it apparent that these substances and their antigenic groups are not products of haphazard degradation of plasma or cellular proteins but products of an organized metabolic process within the epidermal cells. These 5 major epidermal antigens, resolved by electrophoresis and developed by anti-epidermis serum, formed immunoprecipitate arcs in 3 regions of the immunoelectrophoretic pattern; *i.e.*, one in the post-albumin region, two across the β globulins region, one in the β_2 globulin region and one in the post γ globulin region. The nature of 2 of the 5 antigenic substances was indicated by the chemical reactions of their immunoprecipitate arcs: one of the 2 trans β region immunoprecipitate arcs reduced ATZ and stained with greater intensity than the other precipitates, suggesting its higher content of reducible disulfide bonds; and another,

termed β_2 region arc, was oxidized by periodic acid and stained with carbonyl reagents with greater intensity than the other precipitates, suggesting its higher content of 1,2 glycols characteristic of glycoproteins.

By means of SGEP, the same or an even greater number of components were demonstrated than by IEP. The individual substances of most extracts separated neatly into well-defined narrow bands. The number and the distribution of these bands obtained with individual extracts of any one type of epidermis or of horny derivatives were very similar. The identity of the components present in the different extracts, however, could be deduced only by correlating their electrophoretic mobilities and by testing their reaction with sulfhydryl reagents. Applying both criteria, only two substances in the various types of extracts can be considered identical; namely, 1) albumin—being the most anodically advanced sulfhydryl-carrying substance of the albumin zone and 2) one component of the B zone shown to be capable of carrying sulfhydryl groups and of the same electrophoretic mobility as a sulfhydryl-laden component of hyperkeratosis callus (component B_3 of HKK_1). Furthermore, a likeness or an identity among the substances advancing beyond the γ globulin region can be assumed, although specific staining reactions were not found: their cathodic mobility, indicative of their basic nature, sets them apart not only from the anodically migrating components of epidermal origin but also from all plasma proteins.

Through comparison of the data obtained by the two analytic technics and those reported by other investigators, several additional observations can be made regarding the substances of epidermal origin.

The B zone substance of corporeal, palmar and plantar epidermis occasionally separating by SGEP into 2 closely spaced peptides—found to carry sulfhydryl, is most likely responsible for the trans β regions immunoprecipitate arcs—shown to hold the greatest amounts of ATZ-reducing bonds. Because of its electrophoretic mobility, its dual nature and its antigenicity, it appears related to the substance precipitated at pH 4.1 from an extract of plantar callus and termed soluble Keratin A by Matoltsy (11, 30, 31). However, it must be pointed out that in the extracts of hyperkeratosis callus up to 3 components, closely spaced within the B zone, apparently endowed with similar antigenic determinants (producing 1–3 trans β region immunoprecipitate arcs), were found to carry sulfhydryl groups and could therefore qualify for the same designation.

Another β region component was identified and characterized by its reaction with periodic acid in immunoelectrophoretic patterns only. (Starch is an unsuitable medium for reactions involving acid oxidation.) Because of its position in the patterns it is referred to as the β_2 region component. The substance of this component was characterized by an antigenic determinant different from other β region substances and by its sensitivity to oxidation by periodic acid. It was not shown absent in extracts of corporeal epidermis by immunological tests, but its presence there could not be verified by the chemical staining reactions. It was detected in all extracts of palmar and plantar epidermis as well as of callus and ichthyosis scales, suggesting a quantitative relationship to epidermal metabolism characterized by accumulation of horny layer. Whether this substance is a glycoprotein or a polysaccharide was not determined.

By means of SGEP up to 4 substances of differing electrophoretic mobilities were shown to advance against the cathode beyond the γ globulin region, apparently being more basic than any serum protein. Up to 2 of these post γ globulin components were detected by IEP and appeared to share common antigenic sites. Substances of similar electrophoretic mobility were reported present in extracts of psoriatic scales and one was shown antigenically related to a substance of similar mobility present in extracts of hyperkeratosis callus (11). The latter substance was detected among the small molecular substances (est. 25,000) partitioned from an extract of hyperkeratosis callus by column chromatography on a cross-linked dextran (32). Recently, Liss has isolated a crystalline small molecular peptide (27,000) from psoriatic scales (33). This substance was shown by electrophoresis to advance cathodically beyond the γ globulin region and was found antigenically related to a substance of similar electrophoretic mobility present in the extract of callus. Thus, it appears that some of the post

γ globulin substances are antigenically related small molecular peptides, produced in epidermal cells and accumulating in its horny derivatives. Only a trace of the post γ globulin substance was detected in extracts of mucosal epidermis.

One antigenic substance was detected in the albumin region by IEP in all extracts except those of mucosal epidermis. Its antigenic determinant was different from that of normal serum albumin. It was termed post-albumin because of its position in the immunoelectrophoretic pattern. Up to 3 substances resolved within the albumin region by SGEP. These were termed albumin, intra- and post-albumin. Which of the two latter components was responsible for the post-albumin immunoprecipitate arc was not established. The albumin component was immunologically identical with serum albumin. It gave positive reactions with DDD, RSR and ATZ reagents, indicating the presence of bound sulfhydryl groups, whereas the intra- and post-albumin substances did not react at all with any of these reagents. It appears that the albumin-trailing components, present in the greatest amounts in the extracts of palmar and plantar epidermis and of callus and absent in the extracts of mucosal epidermis, are produced by epidermal cells, and accumulate in its horny layers. The studies of Lapresle are worth noting in relation to these data (34): the author has studied the antigenic determinants of peptides produced from human serum albumin by enzymatic hydrolysis and by degradation with extracts of rabbit spleen cells. He found that the latter extract cleaved albumin into 3 antigenic peptides, all of anodic electrophoretic mobility. One of the peptides was endowed with an antigenic determinant different from those of albumin, apparently unmasked by the cleavage.

The electrophoretic mobilities of the albumin-trailing substances in extracts of callus and epidermis are indeed indicative of their relationship with serum albumin, their lack of sulfhydryl groups suggesting that they may represent residual products of albumin after its sulfhydryl groups were utilized in keratin metabolism.

SUMMARY

1. Soluble substances extracted from human corporeal, palmar, plantar and mucosal epidermis and solutes extracted from hyperkeratosis callus and ichthyosis scales were studied by electrophoresis in starch gel and by immunoelectrophoresis. For the latter technic heterologous antisera against human plasma proteins and against human epidermis were used.

2. Five substances of epidermal origin, with different antigenic determinants—immunologically unrelated to those of plasma proteins, were demonstrated in all extracts. These substances appeared to derive from organized epidermal metabolism.

3. The identity and nature of two substances, both of β globulins mobility, was determined by comparing their electrophoretic mobilities, their antigenic relationship and their chemical reactions with sulfhydryl and with carbohydrate reagents. It seems that one of the two is a soluble, sulfhydryl-carrying prekeratin, the other a glucopeptide.

4. The identity of two other types of substances—one of post albumin, the other of post γ globulin mobility—was deduced by noting their positions in the electrophoretic patterns and their immunological likeness; their nature was not determined. The albumin-trailing substances may represent derivatives of serum albumin from which sulfhydryl groups were removed; the post γ globulin substances are small molecular peptides more basic than any serum proteins. Both types were more abundant in cornified epidermis and in its horny derivatives.

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